REMARKS

I. Overview

Claims 1-4, 24, 25, 30 and 31 are pending in the current application. Claims 1-11, 24-26, 30 and 31 were elected by Applicants pursuant to the Examiner's restriction requirement. Claims 5-11 and 26 have additionally been withdrawn based on the Examiner's Office Action dated January 24, 2008 pursuant to 37 CFR 1.142(b) as being drawn to nonelected inventions, without any allowable generic or linking claims.

Claim 30 has been amended. Claims 1-4, 24, 25, 30 and 31 are pending in the current application.

II. Claim Rejections - 35 U.S.C. § 112, second paragraph

Claim 30 is rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner states that claim 30 recites a CNS disorder characterized by extracellular pH, and that one skilled in the art cannot determine what is meant by the phrase. The Examiner further states that perhaps the Applicants intent to refer to "a change" in pH.

Applicants have amended claim 30 by incorporating the Examiner's suggested language, to read: "A method of treating a CNS disorder characterized by a change in extracellular pH in the amygdala comprising: inhibiting the acid-sending ion channel 1 (ASIC1) channel in a patient in need of such treatment." In light of the present amendment, Applicants respectfully request the Examiner to withdraw the §112, second paragraph rejection and place the claims in condition for allowance.

III. Claim Rejections - 35 U.S.C. § 112, first paragraph

A. Enablement

Claims 1-4, 24, 25, 30 and 31 are rejected under 35 U.S.C. § 112, first paragraph, because the Examiner states that the specification does not enable a method of treating anxiety or an anxiety disorder, including *Post-Traumatic Stress Disorder* (PTSD) by administering an ASIC antagonist. The Examiner states the claims recite a method of treating an anxiety disorder in a patient, implying that such a disease is related to the ASIC receptor, without data or references identifying the disease as related to the ASIC receptor. The Examiner continues that the claimed treatments do not flow obviously from the instant disclosure, since no connection was made between the ASIC receptor and underlying mechanisms of anxiety disorders, such as PTSD.

The test for enablement is whether one reasonably skilled in the art would be able to make or use the invention based on "the disclosures in the patent coupled with information known in the art without undue experimention." *U.S. v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988). Additionally, the Federal Circuit has made clear that patents do not need to teach, and should preferably omit, that which is known in the art. *Id.* Further, the MPEP notes that if the statement of utility continas a connotation of how to use, and the art recognizes that standard modes of administration are contemplated, then 35 USC §112 is satisfied. MPEP 2164.01(c).

In the original specification, Applicants defined "anxiety disorder" and explained that PTSD is one of the disorders that is generally characterized as an anxiety disorder along with obsessive compulsive disorder, generalized anxiety disorder as characterized in the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV). [0018]. Applicants' original disclosure recites the connection between anxiety disorders, including the inherently fear-based disoders

such as PTSD, as well as the ASIC receptor and its underlying correlation. The present invention is directed to the effects of ASIC disruption on H+ evoked currents in the brain and more specifically, that the ASIC was found to be present in several areas outside of the hippocampal circuit [0042]. According to the examples, disruption of the ASIC gene eliminated H+ involved currents in the amygdala, a place known in the art to be correlated with PSTD (see attached articles), which involves memory and conditioned fear response. Further the Specification at paragraph 49 details that the discovery of the ASIC channels allows for drugs with inhibit the same to be used as replacements for benzodiazepines, a known drug used in the treatment of PSTD. Finally in the examples section paragraphs 120- 123 specifically describe ASIC1 adnsmygdala –dependant behavior, showing that disruption of ASIC1 disrupted amygdala dependant leraning for fear conditioning. Based on this original support and that known in the art by one having ordinary skill, the relationship between anxiety disorders and the particular disorder of PTSD are sufficiently enabled.

Additionally, Applicants have submitted herewith additional references detailing further work by the inventors and others which confirm the correlation between ASIC receptors and the underlying mechanisms involved in anxiety disorders, notably involving the fear circuit that inherently includes PTSD. The reference *Overexpression of Acid-Sensing Ion Channel 1a in Transgenic Mice Increases Acquired Fear-Related Behavior*, Wemmie et al., PNAS 101(10):3621-3626, March 2004 discloses the abundant expression of ASIC throughout the amygdala complex and the brain regions associated with fear. It further tests the underlying relationship between the channels and the expression of fear-based responses, namely involving PTSD and panic disorder, providing a correlation between the anxiety disorder and the presence of unopposed ASICs. (Wemmie et al. p. 3621). It further provides for the relationship between

mouse behavior, indicating that ASIC1a impacts fear response; wherein overexpressing ASIC1a enhances fear conditioning and eliminating the ASIC1a reduces fear. (Wemmie et al. p. 3625).

Targeting ASIC1a Reduces Innate Fear and Alters Neuronal Activity in the Fear Circuit, Coryell et al., Biol Psych. 2007 sets forth the connection between ASIC receptors and the treatment of anxiety disorders involving fear-modulated responses. Results testing the behavior of cogenic mice, either wild type (ASIC1a+/+) or ASIC1a-null, showed that ASIC1a-null mice had an overall significant reduction in both innate and unconditioned fear. (Coryell et al. p. 3-5). Specifically, when the ASIC1a receptors were knocked-out of mice or were blocked with an antagonizing peptide, there resulted a significant decrease in innate fear of open spaces (Coryell et al. p. 3), reduced unconditioned acoustic startle (Coryell et al. p. 3), reduced fear-based freezing when confronted with a predator's odor, (Coryell et al. p. 3-5).

These references, further confirm the concept of treating an anxiety disorder using an antagonist of the ASIC receptor as described in Applicant's spcifciation. The Examiner's statement that additional enabling experiments are needed to confirm that ASIC receptor antagonists can be administered to treat anxiety disorder such as PTSD, since it is not known if anxiety disorders are caused or made worse by the ASIC receptor, is obviated by the Applicants specification at paragraphs 120-122 which disclosed that ASIC1 disrupted mice had disrupted performance in the elevated plus maze test, a test of baseline fear. Subsequent work by the inventors and others, the Coryell reference provides statistically significant results showing differences in both innate and unconditioned fear-invoking testing environments for mice. Loss of ASIC1a presence and use of an ASIC1a antagonist *in vivo* resulted in the reduction of fear-based behaviors, confirming that, as described in Applicant's specification, that anxiety dirosders such as PTSD may be treated by blocking the receptors with ASIC receptor antagonists.

Applicants respectfully request the Examiner to withdraw the §112, first paragraph rejection and place the claims in condition for allowance.

B. Written Description

Claims 1-3 and 24 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner further states that the absence of sufficient recitation of distinguishing identifying characteristics of the ASIC antagonists, the specification does not provide adequate written description of methods to use the claimed genus.

The specification of the present invention discloses a simple assay which may be performed to identify ASIC channel antagonists at paragraphs 59 and 60. The specification also discloses the family of ASIC cation channels that are gated by reductions in pH and are related to amiloride-sensitive epithelial sodium channels (ENaCs) and the degenerin/mec family of ion channels (DEGs). [0041]. The spefication further provides distinct identifying characteristics of the ion channels that would enable one of ordinary skill in the art to utilize antagonists via any route of administration to utilize the methods of the present invention. For example, the specification provides that subunits of the DEG/ENaC protein family associate as homomultimers and heteromultimers to form voltage-insensitive channels. [0044] Moreover, the individual subunits share a common structure with two transmembrane domains, intracellular carboxyl- and amino-termini, and a large, cysteine-rich extracellular domain thought to serve as a receptor for extracellular stimuli. [0044] Additionally, the specification provides for a specific antagonist of the DEG/ENaC channels, the diuretic amiloride (FDA approved for treatment of

hypertension, all distinguishing chemical and compound characteristics known by one having ordinary skill in the art), and the location and distribution of ASIC in regions of high synaptic plasticity such as the lateral, basolateral and central nuclei of the amygdala that directly implicates the ASIC receptors in the treatment of CNS disorders such as anxiety disorders. [0042, 0051]. Thus the specification gives examples of an assay, examples of identifying characteristics and examples of at least one ASIC1 antagonist, amiloride.

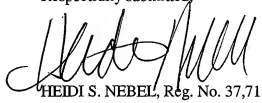
Other ASIC antagonists have been identified by those of skill in the art. The Coryell et al. reference, discloses additional ASIC antagonists. In testing for ASIC1a's impact on the reduction of fear-based freezing of mice when confronted with a predator's odor, the ASIC1a were antagonized using Psalmotoxin (PcTx) containing venom from the tarantula *Psalmapoeus cambridget*, containing a peptide antagonist of ASIC1a channels. (Coryell et al. p. 5). Various forms of antagonists have been obtained and utilized by those of ordinary skill in the art for testing ASIC-related fear in mice, including: (1) unpurified PcTx1 containing venom (Spider Pharm, Yarnell AZ); and (2) purified PcTx1 peptide (Peptides International, Louisville KY). The three-dimensional structure of PcTx in solution and the sequencing of the peptide are known in the art as determined by NMR spectroscopy (see for example, Protein Sci. 2003 Jul;12(7):1332-43 which links recombinant production and solution structure of PcTx1, the specific peptide inhibitor of ASIC1a proton-gated cation channels).

As such it is clear that Applicant's had possession of the methods of treatment embodied in the claims and the disclosure demonstrates the same in compliance with §112 written description. In light of the foregoing, Applicants respectfully request the Examiner withdraw the §112 rejections and place the present claims in condition for allowance.

IV. Conclusion

This is a request to extend the period for filing a response in the above-identified application for one month from April 24, 2008 to May 24, 2008. Applicant is a large entity; therefore, please charge Deposit Account number 26-0084 in the amount of $120.00 to cover the cost of the one month extension. No other fees or extensions of time are believed to be due in connection with this amendment; however, consider this a request for any extension inadvertently omitted, and charge any additional fees to Deposit Account No. 26-0084.

Respectfully submitted,



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Enclosure: articles

1: CNS Spectr. 2003 Sep;8(9):641-50.

Brain-imaging studies of posttraumatic stress disorder.

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Brain-imaging studies of posttraumatic stress disorder (PTSD) have rapidly increased in recent years. Structural studies have identified potential smaller volumes of the hippocampus of traumatized and/or PTSD subjects. Functional activation studies have implicated hyperactive or altered functioning of brain regions, such as the amygdala and the insula, and a failure to engage emotional regulatory structures, such as the medial prefrontal and anterior cingulate cortex. Recent neurochemical investigations have suggested that neuromodulatory systems (eg, gamma-aminobutyric acid, micro-opioid) may underlie these aberrant brain activation patterns. This article reviews the literature on structural, functional, and neurochemical brain-imaging studies of PTSD.

PMID: 15079138 [PubMed - indexed for MEDLINE]

1: Curr Psychiatry Rep. 2003 Oct;5(5):369-83.

Neuroimaging and neurocircuitry in post-traumatic stress disorder: what is currently known?

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Neurobiologic, psychologic, and social factors interact jointly to create and perpetuate the symptoms of post-traumatic stress disorder (PTSD). The fear conditioning paradigm in animal research helped researchers gather preclinical evidence for the possible contribution of several brain areas to PTSD symptoms. In the past 10 years, highly sophisticated neuroimaging techniques made it possible for researchers to look at the brain of patients with PTSD and draw conclusions about the neurocircuitry underlying PTSD symptoms. In this article, the author will review the evidence from neuroimaging studies for the involvement of the following brain areas in PTSD neurocircuitry: the amygdala, the anterior cingulate cortex and subcallosal gyrus, the inferior frontal gyrus, the posterior cingulate cortex, and the hippocampus. Neuroimaging studies have shown these areas as altered in structure or function in patients with PTSD. The author also presents the normal functions that these areas subserve and, whenever possible based on the evidence, infer how their dysfunction may contribute importantly to the symptomatology of PTSD.

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Targeting ASIC1a Reduces Innate Fear and Alters Neuronal Activity in the Fear Circuit

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Background: The molecular mechanisms underlying innate fear are poorly understood. Previous studies indicated that the acid sensing ion channel ASIC1a influences fear behavior in conditioning paradigms. However, these differences may have resulted from an ASIC1a effect on learning, memory, or the expression of fear.

Methods: To test the hypothesis that ASIC1a influences the expression of fear or anxiety independent of classical conditioning, we examined the effects of disrupting the mouse *ASIC1a* gene on unconditioned fear in the open field test, unconditioned acoustic startle, and fear evoked by the predator odor trimethylthiazoline (TMT). In addition, we tested the effects of acutely inhibiting ASIC1a with PcTx, an ASIC1a antagonist in tarantula venom. Our immunohistochemistry suggested ASIC1a is expressed in the bed nucleus of the stria terminalis, medial amygdala, and periaqueductal gray, which are thought to play important roles in the generation and expression of innate fear. Therefore, we also tested whether ASIC1a disruption altered c-fos expression in these structures following TMT exposure.

Results: We found that the loss of ASIC1a reduced fear in the open field test, reduced acoustic startle, and inhibited the fear response to TMT. Similarly, intracerebroventricular administration of PcTx reduced TMT-evoked freezing in ASIC1a$^{+/+}$ mice but not ASIC1a$^{-/-}$ mice. In addition, loss of ASIC1a altered TMT-evoked c-fos expression in the medial amygdala and dorsal periaqueductal gray.

Conclusions: These findings suggest that ASIC1a modulates activity in the circuits underlying innate fear. Furthermore, the data indicate that targeting the ASIC1a gene or acutely inhibiting ASIC1a suppresses fear and anxiety independent of conditioning.

Key Words: Acid sensing ion channels, anxiety, amygdala, anxiolytics, fear, periaqueductal gray

While some fears are learned through association, humans and animals may also be biologically or innately predisposed to fear some stimuli (1–4). This predisposition may contribute to neuroticism and the development of anxiety disorders (3,4). Thus, understanding the mechanisms of innate fear in animal models may provide valuable insight into the pathogenesis of anxiety disorders.

Innate (or unconditioned) fear is poorly understood, however some required brain regions have been identified in rodents. Inactivating the bed nucleus of the stria terminalis (BNST) (5) or medial amygdala (6) attenuated predator odor-evoked fear, and other forms of unconditioned fear (5,7). Thus, it has been suggested that unconditioned fear may rely on brain circuits different from those required for fear conditioning such as the lateral, basolateral, and central amygdala (8,9). However, evidence also suggests overlap between conditioned and unconditioned fear circuits. For example, inactivating the bed nucleus of the stria terminalis (10) and medial amygdala (11) also attenuated context fear conditioning. Furthermore, inactivating the basolateral amygdala (BLA) inhibited unconditioned fear (6,12) in addition to suppressing the acquisition, retention, and expression of fear conditioning (for review [13]). Beyond the anatomic basis for unconditioned fear, relatively little is known about the molecular mechanisms underlying innate fear.

A molecule that might contribute to innate fear is the acid sensing ion channel ASIC1a. ASIC1a is a recently discovered member of the degenerin/epithelial Na^+ channel family that is activated by extracellular acidosis and is expressed in the amygdala complex and elsewhere in the brain (14–17). ASIC1a is required for acid-evoked currents in central neurons, and is located at excitatory synapses (14), where it modulates synaptic Ca^{2+} signaling (18,19) and synaptic plasticity (14, 20–22). ASIC1a enables dendritic spines to respond to acid pH (18), suggesting synaptically released protons might activate it (23). ASIC1a can be modulated by N-methyl-D-aspartate (NMDA)-receptor activation (19), and the loss of ASIC1a disrupted NMDA-receptor dependent long-term potentiation and temporal summation of excitatory postsynaptic potentials (14). Consistent with a role in fear learning and memory, deleting ASIC1a reduced fear in conditioning paradigms (17), while overexpressing ASIC1a increased context fear conditioning (24). However, it is not clear to what degree the ASIC1a-dependent effects were due to differences in fear acquisition, fear retention, or fear expression.

To test the effects of ASIC1a on the expression of fear and anxiety, we examined the impact of disrupting the *ASIC1a* gene or acutely blocking ASIC1a on mouse models of unconditioned fear, including fear of open spaces, unconditioned acoustic startle, and fear evoked by the predator odor trimethylthiazoline (TMT) (7,8,25–28). To explore whether differences were due to sensory or motor dysfunction, we also tested the effects of ASIC1a on hearing, footshock evoked startle, and olfactory dependent behaviors. Finally, we examined ASIC1a distribution in forebrain and midbrain structures required for TMT-evoked fear, and tested whether loss of ASIC1a altered c-fos induction in these structures.

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Methods and Materials

Mice

Congenic (C57Bl6) ASIC1a$^{+/+}$ and ASIC1a$^{-/-}$ mice were produced as described previously (14). Mice received standard chow (LM-485; Teklab, Madison, Wisconsin) and water ad libitum. Animal care met National Institutes of Health standards and all procedures were approved by the University of Iowa Animal Care and Use Committee. Separate naive mice were used for each behavioral protocol. All behavioral scoring was performed by experimenters blinded to genotype. Comparison groups were matched for gender and age (range, 8–24 weeks). Except where specified, statistical significance was assessed using analysis of variance (linear and mixed models) with or without repeated measures. Also, where appropriate a *t* test was used to assess two-sample comparisons; $p < .05$ was considered significant.

Open Field Activity

Mice were placed in a 40.6 × 40.6 × 36.8 cm open-field (San Diego Instruments, San Diego, California), 55 lux, for 5 min. Center activity was defined as beam breaks occurring in the center (15.2 × 15.2 cm).

Acoustic Startle and Evoked Auditory Brainstem Responses

Startle was measured using an SR-LAB (San Diego Instruments) 860 lux. Following a 3-min acclimation, the startle response was assessed in response to 10, 50 msec, 115 dB acoustic startle stimuli, with an intertrial-interval of 5–60 sec with constant white background noise (58–dB). Mice ranged from 9–21 weeks. Weight was assessed at time of testing (+/+ ave. = 25.4 g ± 3.68 SD; −/− ave. = 25.2 g ± 3.69 SD). Footshock-evoked startle was assessed in response to five footshocks (.7 mA, 500 msec) with an inter-shock interval between 30 and 60 sec. Evoked auditory brainstem responses (EABR) was done as described previously (29). EABR attenuation threshold was defined as the maximum sound attenuation (dB) at which an identifiable wave I peak of the EABR was still observed.

Predator Odor Evoked Behavior

Mice were placed in an acrylic glass chamber (18 cm × 18 cm) with a beaker containing TMT (30 μL) (PheroTech, Delta, British Columbia, Canada), butyric acid (105 μL) (Sigma-Aldrich, St. Louis, Missouri), or water. Freezing was defined as the absence of movement except for respiration and avoidance was defined as the amount of time an animal was not touching or climbing on the beaker. Statistical significance of avoidance was assessed by chi-square analysis of least squares means estimates from a negative binomial regression.

Olfactory Tests

Food detection was assessed similar to a previous approach (30). Food was withheld overnight, and bacon (1 cm × 1 cm) or a similar sized piece of red plastic was buried 1 cm beneath bedding in a clean home cage. The latency to find and uncover the object was recorded. Bedding preference was assessed similar to a previous protocol (31). Bedding was placed into the corners of a square open field (60 × 60 cm) into caps from 4–50 mL falcon tubes. Three of the corners had clean bedding and the remaining corner had bedding from the home cage of a nonlittermate mouse of the opposite sex. Used bedding was rotated to different corners in a pseudo-random order between test subjects. Time spent in each of four quadrants (10 × 10 cm surrounding the bedding samples) was scored.

Whole-Cell Voltage-Clamp Recordings

Cortical neurons were obtained from 1–2 day old pups and cultured for 8–14 days as described (15,17). Whole cell voltage-clamp recordings were obtained at 20–23°C using an Axopatch 200B amplifier (Axon Instruments, Hawthorn East, Victoria, Canada) and Clampex 8.2 (Axon Instruments) sampled at 200 μsec interval and filtered at 2 kHz. Extracellular pH was changed with a rapid solution changer (RSC-200; Biologic, Claix, France). Membrane voltage was maintained at -70 mV. Bath solutions contained (in mM): 100 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 MES and pH was adjusted with TMA-OH. Patch pipettes (3–5 MΩ) contained (in mM): 10 NaCl, 70 K-gluconate, 10 KCl, 1 $MgCl_2$, 10 EGTA, 25 HEPES, and 3 Na_2ATP, adjusted to pH 7.3 with KOH. PcTx venom (Spider Pharm, Yarnell, Arizona) was diluted in the bath solutions (final concentration 1.8 ng/μL).

PcTx Venom Administration

Intracerebroventricular (ICV) guide cannulae were implanted into the left lateral ventricle of anesthetized mice (.03 mm caudal, .1 mm lateral, .3 mm ventral with respect to bregma). Cannulae were fixed to the skull with dental cement and an anchoring screw. Two to four days later, 5 μL PcTx venom (9 ng/μL) in sterile artificial cerebrospinal fluid (ACSF) (in mM: NaCl 124, KCL 3, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 26) or ACSF alone was injected by hand (over 60 sec) using a 10 μL-Hamilton syringe and PE10 tubing connected to a 30-gauge stainless steel injector. Mice were returned to the home cage for 5 min; then, TMT-evoked behavior was assessed. The response to TMT was tested on consecutive days. ACSF was injected on days 1 and 3 and PcTx was injected on day 2. Because the TMT responses on day 1 and day 3 were not different, these data were averaged within animal comparisons between PcTx and ACSF. Cannula placement was verified by methylene blue injection. Animals with misplaced or plugged cannula were excluded from the analyses.

ASIC1 Immunohistochemistry

The protocol is similar to that described (17), with changes that improved signal to noise, including use of the CryoJane sectioning protocol (Electron Microscopy Sciences, Hatfield, Pennsylvania), and extended antibody incubation and blocking times. Brains were frozen on dry ice and embedded in optimal cutting temperature compound (Electron Microscopy Sciences). Coronal slices (10 μm) were cut and mounted on slides and postfixed in phosphate buffered saline (PBS) with 4% formaldehyde and 4% sucrose for 10 min, followed by .25% triton X-100 in PBS for 5 min at 23°C, and then incubated in affinity purified ASIC1a antibody (17) (1:50) for 24 hours at 4°C. Following PBS wash, sections were incubated with Cy3-coupled anti-rabbit IgG (Jackson ImmunoResearch Labs, West Grove, Pennsylvania) 1:200 for 1 hour, 23°C. Slices were visualized with a Zeiss epifluorescence microcope (LSM 510 Meta; Carl Zeiss MicroImaging, Inc., Thornwood, New York). Images were captured at 10× magnification and compiled using the tiling software function (Zeiss) to create composite image of coronal hemi-sections.

C-fos Immunohistochemistry

One hour following TMT exposure (in the home cage), mice were euthanized with halothane and perfused pericardially with PBS followed by 4% paraformaldehyde. Brains were extracted, postfixed in 4% paraformaldehyde, and cryoprotected in 30% sucrose. Cryostat sections (40 μm) were cut from anterior to posterior and c-fos labeled cells from the first four consecutive intact tissue sections from a given structure for each mouse were

quantified by an investigator blinded to genotype using Image J software (National Institutes of Health, Bethesda, Maryland) as described (32). The anatomical boundaries for the structures tested were defined based on the Paxinos atlas (33). For amygdala nuclei (central, lateral [LA], and medial) sections were selected from between 1.7 and 1.6 mm caudal to bregma. For the BNST and piriform cortex, sections were collected from between .14 and .28 mm rostral to bregma. For periaqueductal gray (PAG) analysis, sections were collected from between 4.48 and 4.60 mm caudal to the bregma.

Results

Loss of ASIC1a Reduces Fear-Behavior in the Open Field

To test whether ASIC1a disruption affects innate fear, we assessed fear in the open field by measuring infrared beam breaks. Naive mice fear open spaces (27), likely because of the associated risk of predation. Thus, in this test mice usually avoid the center, preferring to move around the edges. Consistent with a fear of open spaces, we found that both wild-type and ASIC1a-null mice avoided the center. However, ASIC1a-null mice had significantly more center beam breaks than wild-type controls (Figure 1A). Total beam breaks were similar between the two genotypes (Figure 1B), suggesting that loss of ASIC1a does not affect motor activity overall. These data suggest that the loss of ASIC1a reduces fear of open spaces.

ASIC1a Disruption Reduces Unconditioned Acoustic Startle, but Does Not Affect Hearing

The startle response to sudden noise is thought to reflect emotional arousal and is increased in patients with anxiety disorders (34–36). Particularly in mice, baseline startle may be a valuable measure of anxiety (25). Therefore, we hypothesized that if ASIC1a contributes to innate fear, then disrupting ASIC1a may attenuate unconditioned acoustic startle. To test this hypothesis, we used an approach similar to that described by others (25); we placed naive wild-type and ASIC1a-null mice into an acoustic startle chamber and assessed the average maximal startle amplitude evoked by 10 presentations of white noise (115 dB). Consistent with reduced innate fear, the ASIC1a$^{-/-}$ mice startled significantly less than ASIC1a$^{+/+}$ mice (Figure 1C, 1D). In addition, the differences between the two groups did not vary significantly by trial number (Figure 1C), suggesting the effect of ASIC1a on acoustic startle was probably not due to altered sensitization or habituation.

To test whether the blunted acoustic startle in the ASIC1a$^{-/-}$ mice might be caused by hearing loss, we also assessed EABR. We found no differences in the EABR attenuation threshold between ASIC1a$^{+/+}$ and ASIC1a$^{-/-}$ mice (Figure 1E), suggesting hearing is normal in the ASIC1a-null mice. We also examined whether ASIC1a disruption might impair motor reflexes. We assessed startle responses to a footshock (.7 mA), which is well above the threshold of detection (17,24). Because footshock activates peripheral sensory and nociceptive fibers, spinal reflexes are likely to play a large role in this behavior (37). We found ASIC1a$^{+/+}$ and ASIC1a$^{-/-}$ mice produced a similar response to footshock (ASIC1a$^{+/+}$ amplitude = 1774.5 ± 247. (SEM); ASIC1a$^{-/-}$ amplitude = 1645.8 ± 201.7 (SEM); $n = 5$ per group; df (8), t = .37, p = .74). This result is consistent with previous indications that sensory responses and motor function are grossly intact in the ASIC1a$^{-/-}$ mice (14,17,38,39).

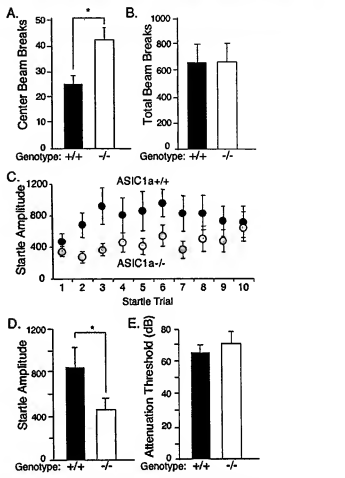

Figure 1. ASIC1a disruption reduces center-avoidance in the open field and reduces acoustic startle. **(A)** Center avoidance was measured by center beam breaks. ASIC1a$^{-/-}$ mice had significantly more center beam breaks than ASIC1a$^{+/+}$ mice ($p < .004$; +/+, $n = 13$; −/−, $n = 16$). **(B)** Total activity in the open field was similar between genotypes (df (30), $t = .04$, $p > .96$; +/+, $n = 9$; −/−, $n = 12$). **(C)** Unconditioned acoustic startle amplitude separated by trial number. There was no significant genotype × trial interaction (+/+, $n = 9$; −/−, $n = 12$), (df (1,9), $F = 1.43$, $p = .18$) suggesting the differences between the genotypes did not vary significantly between trials. **(D)** Amplitude of acoustic startle responses pooled from 10 trials was reduced in the ASIC1a$^{-/-}$ mice relative to ASIC1a$^{+/+}$ controls (df (19), $t = 2.00$, $p = .029$). **(D)** Attenuation threshold of the evoked auditory brainstem response did not differ between ASIC1a$^{+/+}$ and ASIC1a$^{-/-}$ mice (df (11), $t = -1.38$, $p = .20$; +/+, $n = 6$; −/−, $n = 7$).

Loss of ASIC1a Disrupts Unconditioned Responses to the Predator Odor TMT

As another model of innate fear we assessed TMT-evoked fear behaviors. TMT is a sulfur-containing compound isolated from the anal gland of foxes (40) and previously shown by others to evoke BNST-dependent unconditioned freezing in rodents (5,41–43). Consistent with those studies, TMT evoked significant freezing in ASIC1a$^{+/+}$ mice. In contrast, ASIC1a$^{-/-}$ mice froze significantly less in response to TMT (Figure 2A, 2B) and see Supplement 1 online). Consistent with the previous suggestion that female C57Bl6 mice may have greater predator odor-evoked fear (44), wild-type females froze more than their male counterparts (Figure 2C). However, the loss of ASIC1a reduced freezing in both genders. In addition to their reduced freezing response, the ASIC1a$^{-/-}$ mice avoided contact with the TMT less than wild-type

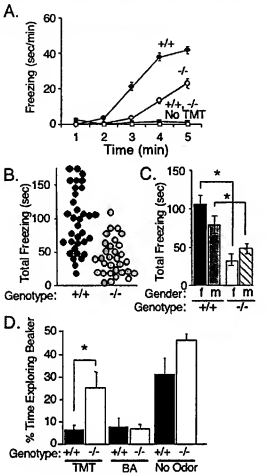

Figure 2. ASIC1a disruption reduces TMT-evoked fear behaviors. **(A)** Freezing response to TMT during 5 min. trial. The two genotypes had nonparallel response profiles to TMT over time (genotype × min interaction, $p = .0066$) and ASIC1a$^{+/+}$ mice exhibited significantly more freezing at min 3, 4, 5 (all p values < .0001; ASIC1a$^{+/+}$, $n = 31$; ASIC1a$^{-/-}$ $n = 30$). **(B)** Scatter plot of TMT-evoked freezing over 5-min trial. **(C)** TMT evoked more freezing in female (f) wild-type mice than in their male (m) counterparts (female +/+ ($n = 15$) versus male +/+ ($n = 18$); df (31), $t = 2.02$, $p < .03$). ASIC1a disruption significantly reduced freezing in both genders (female +/+ ($n = 15$) versus female −/− ($n = 16$); df (29), $t = 6.30$, *$p < .0001$) (male +/+ ($n = 18$) versus male −/− ($n = 15$); df (31), $t = 2.23$, *$p < .02$). **(D)** Wild-type mice spent significantly less time exploring the TMT container than did ASIC1a$^{-/-}$ mice (*$p = .03$; ASIC1a$^{+/+}$, $n = 7$; ASIC1a$^{-/-}$, $n = 7$), although both genotypes avoided BA to a similar degree ($p = .54$; +/+, $n = 8$; −/−, $n = 8$). The ASIC1a$^{-/-}$ mice tended to explore the beaker more in the absence of exogenous odor, but the effect was not statistically significant ($p = .09$). BA, butyric acid; TMT, trimethylthiazoline.

controls (Figure 2D). Freezing was specific to TMT since neither genotype froze in response to butyric acid (BA), a pungent non-predator odor (not shown). Taken together these data suggest ASIC1a contributes to unconditioned fear evoked by TMT.

To test whether the impaired response to TMT was due to aberrant olfaction, we assessed several olfactory dependent behaviors. We found ASIC1a$^{+/+}$ and ASIC1a$^{-/-}$ mice avoided BA to an equivalent degree (Figure 2D). In addition, ASIC1a$^{-/-}$ mice used olfactory cues appropriately to find buried food (Figure 3A). We also assessed preference for investigating bedding used by nonlitter mate mice of the opposite sex, previously shown to depend on pheromonal olfaction (45). ASIC1a-null mice showed a similar preference for used bedding to that of wild types (Figure 3B). To further assess olfaction in ASIC1a-null mice, we examined the ability of TMT to induce c-fos expression in the olfactory (piriform) cortex. We found that ASIC1a-null mice expressed normal levels of c-fos in the piriform cortex in response to TMT (Figure 3C). These behavioral and functional data suggest that ASIC1a-null mice have grossly normal olfaction and suggest that olfactory defects are unlikely to explain the severe deficits in the TMT response.

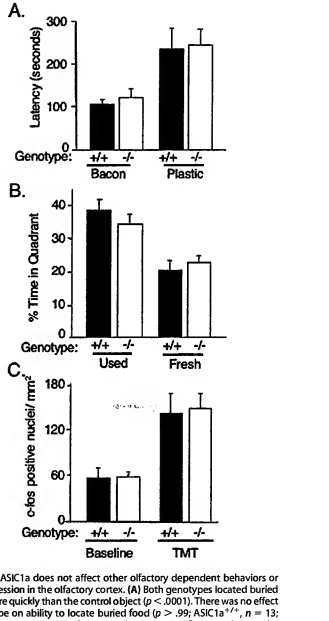

Figure 3. ASIC1a does not affect other olfactory dependent behaviors or c-fos expression in the olfactory cortex. **(A)** Both genotypes located buried bacon more quickly than the control object ($p < .0001$). There was no effect of genotype on ability to locate buried food ($p > .99$; ASIC1a$^{+/+}$, $n = 13$; ASIC1a$^{-/-}$, $n = 14$). **(B)** Both groups showed a significant preference for used bedding versus fresh bedding ($p < .0001$). There was no effect of genotype on bedding preference (df (1, 17.2), $F = 1.19$, $p > .29$; ASIC1a$^{+/+}$, $n = 7$; ASIC1a$^{-/-}$, $n = 8$). **(C)** Trimethylthiazoline evoked significant c-fos immunostaining in the olfactory (piriform) cortex compared to baseline controls (df (1,10), $F = 26.43$, $p = .0004$); (ASIC1a$^{+/+}$, TMT, $n = 5$; no TMT, $n = 4$), (ASIC1a$^{-/-}$, TMT, $n = 5$; No TMT, $n = 4$), but there was no interaction between genotype and TMT exposure (df (1,10), $F = .02$, $p > .89$). TMT, trimethylthiazoline.

Acute Inhibition of ASIC1a Decreases TMT-Evoked Freezing

Our data indicate that genetically disrupting ASIC1a reduces unconditioned fear. This might be due to a requirement for ASIC1a during fear behavior or a result of abnormal development. To distinguish between these possibilities we tested whether acute inhibition of ASIC1a could reduce fear behavior using PcTx venom from the tarantula *Psalmapoeus cambridgei*. Previous studies indicated that PcTx venom contains a peptide antagonist of ASIC1a channels (46) and that ICV infusion of PcTx reduces acid-associated toxicity during stroke (16). Consistent with previous studies (16,47), in cultured cortical neurons from ASIC1a$^{+/+}$ mice, PcTx (1.8 ng/μL) blocked 64% (± 6% SEM, n = 3) of the average peak pH 6-evoked current (Figure 4A). Because PcTx only blocks ASIC1a homomultimeric channels, the remaining current is likely to be due to ASIC1a-containing heteromultimers with ASIC2, as suggested previously (47). We hypothesized that a similar dose of PcTx venom (5 μl, 9 ng/mL) in ACSF would reduce unconditioned fear of the predator odor TMT. ICV administration of PcTx venom reduced TMT-evoked freezing in wild-type mice, but did not affect TMT-evoked freezing in ASIC1a-null mice (Figure 4B). The ASIC1a-null mice provided an important control for nonspecific effects of the venom. These results suggest that acutely inhibiting ASIC1a reduces unconditioned fear. In addition, these data rule out a developmental requirement for ASIC1a in innate fear, and suggest that innate fear is closely tied to ASIC1a function.

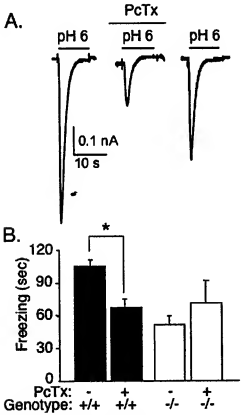

Figure 4. Inhibiting ASIC1a with PcTx reduces the freezing reponse to TMT. **(A)** Consistent with previous studies (16, 47), PcTx (9 ng/μL) inhibited acid-evoked currents in cultured cortical neurons. Elapsed time between traces was 110 and 160 sec respectively. **(B)** ICV infusion of PcTx significantly reduced TMT-evoked freezing during a 5 min trial in ASIC1a$^{+/+}$ mice (df = 26.9, t = −3.31, *p = .0027; n = 12) but not in ASIC1a$^{-/-}$ mice (df = 26.9, t = 1.29, p > .20; n = 7). ICV, intracerebroventricular; TMT, trimethylthiazoline.

ASIC1a is Abundant in the BNST, Amygdala, and Periaqueductal Gray

Earlier studies suggested that ASIC1a is enriched in the amygdala (17), including the medial nucleus which is required for predator odor evoked fear (26). However, it is not known whether ASIC1a is also expressed in the BNST or PAG which play critical roles in the expression of innate fear, freezing, and other defensive behaviors (7,48–54). Therefore, to assess ASIC1a distribution in these key structures, we used immunohistochemistry to test ASIC1a expression in the forebrain and midbrain. ASIC1a$^{-/-}$ mice provided a valuable control for ASIC1a-specific immunostaining. Relative to other structures, we found ASIC1a immunostaining was strikingly abundant in the BNST, BLA, and PAG of ASIC1a$^{+/+}$ mice (Figure 5A, 5B, 5C). Consistent with our previous results (17), ASIC1a was also present in the lateral, medial, and central amygdala nuclei as well as the cingulate cortex, habenula, and lateral hypothalamus (Figure 5A). These data suggest ASIC1a may contribute to innate fear by influencing neuronal activity in fear circuit structures.

Loss of ASIC1a Alters TMT Induced c-fos Expression in the Medial Amygdala and Dorsal Periaqueductal Gray

To assess whether the differences in fear behavior were associated with changes in fear circuit activity, we assessed expression of the immediate early gene c-fos as a marker of neuron activation in key fear circuit structures following TMT exposure. We hypothesized that ASIC1a disruption would alter c-fos expression in the medial amygdala, BNST and PAG; regions where ASIC1a is abundant and that are critical for TMT-evoked fear (5,6,50,55–57). We also assessed c-fos expression in the LA and BLA because ASIC1a is abundant in these structures (17). However, the importance of the LA and BLA in TMT-evoked fear is less certain (6,8,43). Following TMT exposure, we detected ASIC1a-dependent differences in both the medial amygdala and dorsal PAG (Figure 6). In the medial amygdala, the loss of ASIC1a attenuated c-fos induction (Figure 6A, 6B, 6E). Interestingly, in the dorsal PAG (dPAG), TMT produced differential effects in ASIC1a$^{+/+}$ mice compared to ASIC1a$^{-/-}$ mice. In ASIC1a$^{+/+}$ mice, TMT reduced c-fos expression in the dPAG; whereas in the ASIC1a$^{-/-}$ mice, TMT marginally increased c-fos expression (Figure 6E). Because of the importance of the medial amygdala and PAG in freezing and defense behaviors, these differences are likely to contribute to the reduced fear behaviors in the ASIC1a$^{-/-}$ mice.

In contrast, we found no significant ASIC1a-dependent differences in the BNST, ventral PAG, BLA or LA following TMT exposure (Figure 6F). This could mean that ASIC1a in these regions does not contribute to TMT-evoked fear. It is also possible that c-fos expression is not sensitive to ASIC1a-dependent effects in these regions.

Discussion

Relatively little is known about the molecular underpinnings of innate fear (7,8), however, our results suggest that ASIC1a plays a critical role. In naive mice, loss of the *ASIC1a* gene or acute ASIC1a inhibition reduced fear-related behaviors evoked by multiple stimuli and measured by multiple behavioral outputs, including avoidance, startle, and freezing. Together these data suggest that ASIC1a plays a central role in innate fear, and that

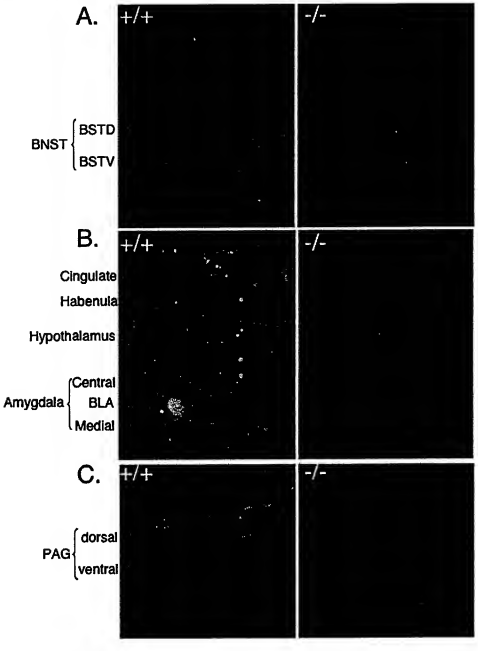

Figure 5. ASIC1a immunohistochemistry in the forebrain and midbrain of ASIC1a$^{+/+}$ and ASIC1a$^{-/-}$ mice. **(A)** In the forebrain, ASIC1a expression is enriched in the dorsal and ventral BNST relative to nearby structures. **(B)** ASIC1a is also abundant in the amygdala, particularly in the BLA, but also in the central and medial nuclei. In addition, ASIC1a is enriched in the cingulate cortex, habenula, somatosensory cortex, and lateral hypothalamus. **(C)** In the midbrain, ASIC1a is enriched in the dorsal and ventral PAG. BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; PAG, periaqueductal gray.

understanding ASIC1a function may provide important insights into the underlying neurobiology.

The fear reducing effects of inhibiting ASIC1a with PcTx have several implications. First, PcTx was administered by ICV cannula, therefore these data suggest a central nervous system site of ASIC1a action. Second, because PcTx reduced freezing acutely, our data suggest that channel activity is necessary for normal fear behavior independent of brain maturation or development. Protons are the only known activator of ASIC1a (20–22,58), suggesting the possibility that pH fluctuations occur in the fear circuit. A possible source of extracellular protons during fear behaviors is from neurotransmitter release; synaptic vesicles are acidic and release their contents into the synaptic cleft during neurotransmission (23). Other causes of interstitial acidosis might also activate ASIC1a and increase fear. Alternatively, ASIC1a could be activated by ligands not yet identified. Third, PcTx does not block other ASICs, nor does it block ASIC1a-containing heteromultimeric channel complexes (47). Therefore, the fear reducing effects of PcTx also point to a role for ASIC1a homomultimeric channels in innate fear.

Our behavioral, localization and c-fos data suggest that ASIC1a modulates activity in the fear circuit. ASIC1a is expressed at numerous locations in the fear circuit including the BNST, medial amygdala, basolateral amygdala, and PAG, and loss of ASIC1a altered c-fos expression in both the medial amygdala and the PAG. These structures are thought to play critical roles in innate fear (5,7,28,50,55,56,59). However, there are limitations to using c-fos as a marker of neuronal activity. First, c-fos may not detect activity in all neurons (60). Therefore, our studies might have missed ASIC1a-dependent differences in some brain struc-

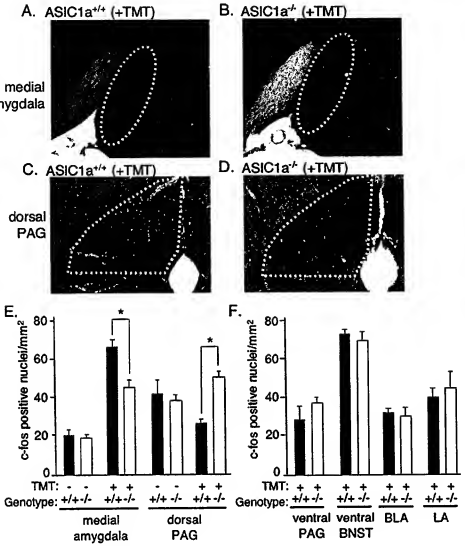

Figure 6. Loss of ASIC1a alters TMT induction of c-fos in the medial amygdala and dPAG. **(A, B)** Examples of c-fos expression in the medial amygdala following TMT exposure from ASIC1a$^{+/+}$ and ASIC1a$^{-/-}$ mice. **(C, D)** Examples of c-fos expression in the dPAG following TMT exposure. **(E)** Quantification revealed a significant increase in c-fos expression in medial amygdala following TMT exposure (df (1, 17.5), F = 128.85, $p < .0001$) as well as a significant genotype × TMT interaction (df (1, 17.5), F = 8.82, p = .0084; No odor (ASIC1a$^{+/+}$, n = 5; ASIC1a$^{-/-}$, n = 5); TMT (ASIC1a$^{+/+}$, n = 10; ASIC1a$^{-/-}$, n = 9)). In the dPAG, TMT decreased c-fos in the dPAG of ASIC1a$^{+/+}$ mice (df = 15, t = −2.91, p = .011) and increased c-fos expression in the dPAG of ASIC1a$^{-/-}$ mice, although the p value was marginal (df = 19, t = −1.99, p = .06). Consequently, following TMT exposure c-fos expression in the dPAG was significantly greater in ASIC1a$^{-/-}$ mice relative to ASIC1a$^{+/+}$ mice (df = 28, t = −6.40, $p < .0001$), resulting in a significant genotype × TMT interaction (df (1, 34), F = 11.13, p = .002; No odor (ASIC1a$^{+/+}$, n = 4; ASIC1a$^{-/-}$, n = 4); TMT (ASIC1a$^{+/+}$, n = 13; ASIC1a$^{-/-}$, n = 17). **(F)** In the BNST, vPAG, BLA, and LA there were no significant differences between genotypes in c-fos expression following TMT exposure (BNST: df = 6.66, t = .68, $p > .51$; ASIC1a$^{+/+}$, n = 6; ASIC1a$^{-/-}$, n = 6) (vPAG: df = 7, t = −1.2, p = .24; ASIC1a$^{+/+}$, n = 4; ASIC1a$^{-/-}$, n = 5) (BLA: df = 10, t = .22, $p > .83$; n = 6 per group) (LA: df = 10, t = −.59, $p > .56$; n = 6 per group). BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; dPAG, dorsal periaqueductal gray; LA, lateral; TMT, trimethylthiazoline; vPAG, ventral preiaqueductal gray.

tures. Second, the neural circuitry of the medial amygdala and PAG are complex (50,53); making it difficult to know whether the ASIC1a-dependent differences occur in excitatory or inhibitory pathways.

Others have previously examined c-fos induction by TMT (55,61) and cat odor (62). In general, those studies found c-fos was increased at multiple locations in the fear circuit; although some differences between TMT and cat odor have been pointed out (26,28) which could reflect differences between specific odors, odor concentration, or other testing conditions. Our data are consistent with the suggestion that predator odors increase c-fos in the fear circuit, particularly in the ventral BNST and the medial amygdala (55,62). Interestingly, cat odor was previously suggested to increase c-fos in the dPAG (62), while in our studies TMT suppressed c-fos in the dPAG of wild type mice. This difference could be due to the testing conditions. In our studies the mice were unable to escape TMT and exhibited robust freezing, while in the previous study rats were given a hide box to escape the cat odor (62). These observations are consistent with the previously observed role of the dPAG in escape (53), and with others' data suggesting an inverse correlation between freezing and c-fos expression in the dPAG (63). Thus, the altered activities in the medial amygdala and dPAG observed here are likely to contribute to the reduced fear and reduced freezing in the ASIC1a$^{-/-}$ mice.

Based on these studies, one might expect ASIC1a to affect anxiety-related behavior in the elevated plus maze (EPM). Curiously, open arm crossings and risk assessing behaviors in a 1-trial EPM paradigm were previously shown to be unaffected by ASIC1a-disruption (17). Because EPM can be sensitive to a number of factors including handling, lighting, and genetic background (64), it is possible that small ASIC1a-dependent effects on EPM may have been missed (17). Alternatively, ASIC1a may not contribute to this behavior. Others have noted that 1-trial EPM is not universally sensitive to manipulations that affect fear, including basolateral amygdala lesions (65), and altered neuropeptide signaling (64).

Here we found that the loss of ASIC1a reduces fear behaviors thought to occur independent of associative learning. Hence, our results suggest that ASIC1a modulates fear circuit activity independent of its role in synaptic plasticity (14). Perhaps the previously observed effects of ASIC1a on conditioned fear tasks (17,24) are due to differences in fear expression rather than fear conditioning. It is interesting to note that AMPA receptor block-

ade (9) and in some cases NMDA receptor blockade inhibit the expression of both conditioned and unconditioned fear (66,67) in addition to inhibiting fear learning (13). Thus, the relationship between innate and conditioned fear may be more similar than is generally believed. Further studies will be needed to better understand the role of ASIC1a on synaptic transmission and plasticity in the fear circuit.

Finally, our data may have important clinical implications. The effects of ASIC1a on innate fear raise the possibility that mutations affecting ASIC1a expression or function may influence the heritability of neuroticism, phobias, and other anxiety traits. Consistent with a possible role for ASIC1a in human anxiety, a genomic study linked a region containing the ASIC1a gene (Chr 12q13) to panic disorder with agoraphobia (68). Our studies suggest ASIC1a might also be targeted for treatment. The acute effects of PcTx on the expression of innate fear suggest that blocking ASIC1a could attenuate anxiety symptoms that arise independent of prior experience. Furthermore, because ASIC1a expression is abundant in the fear circuit, ASIC1a antagonists might preferentially reduce fear relative to other brain functions. In addition, the rapid effects of blocking ASIC1a might be an advantage over current treatments, some of which can take weeks to alleviate symptoms. Together these observations underscore the therapeutic potential for targeting ASIC1a in anxiety disorders.

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Supplementary material cited in this article is available online.

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Overexpression of acid-sensing ion channel 1a in transgenic mice increases acquired fear-related behavior

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The acid-sensing ion channel 1a (ASIC1a) is abundantly expressed in the amygdala complex and other brain regions associated with fear. Studies of mice with a disrupted *ASIC1* gene suggested that ASIC1a may contribute to learned fear. To test this hypothesis, we generated mice overexpressing human ASIC1a by using the pan-neuronal synapsin 1 promoter. Transgenic ASIC1a interacted with endogenous mouse ASIC1a and was distributed to the synaptosomal fraction of brain. Transgenic expression of ASIC1a also doubled neuronal acid-evoked cation currents. The amygdala showed prominent expression, and overexpressing ASIC1a enhanced fear conditioning, an animal model of acquired anxiety. These data raise the possibility that ASIC1a and H^+-gated currents may contribute to the development of abnormal fear and to anxiety disorders in humans.

Anxiety disorders such as posttraumatic stress disorder and panic disorder can cause significant distress and are frequently disabling (1). Pavlovian fear conditioning is an important animal model of anxiety with both anatomical and physiological parallels to anxiety disorders in humans (2–5). Thus, understanding the mechanisms that underlie fear conditioning might offer the opportunity for new treatment and prevention strategies for these debilitating illnesses. Our recent data suggest that acid-sensing ion channels (ASICs) may play a role in fear conditioning (6).

ASICs are neuronally expressed members of the degenerin/epithelial Na^+ channel family (for review, see refs. 7–10). ASIC subunits form homomultimeric and heteromultimeric channel complexes that are activated by a fall in extracellular pH. Central neurons express three ASIC subunits (ASIC1a, ASIC2a, and ASIC2b, where a and b refer to splice variants) (11–15). ASIC1a appears to play a prominent role in determining current amplitude and also affects the kinetics of H^+-gated current (14, 16–18). ASIC2a modulates desensitization, recovery from desensitization, pH-sensitivity, and the response to modulatory agents such as Zn^{2+} and FMRFamide (19–23). The role of ASIC2b is uncertain, although in the absence of ASIC1a it inhibits ASIC2a-mediated current (12, 17).

Earlier work showed ASIC1a is expressed throughout the brain, with prominent expression in areas that receive rich synaptic input (6, 11, 14, 24). Endogenous ASIC1a was enriched in synaptosome-containing brain fractions, and ASIC1a transfected into neurons appeared in the cell body and colocalized with the postsynaptic density 95 protein (PSD-95) at synapses (16). Consistent with a role in synaptic physiology, targeted disruption of the *ASIC1* gene in mice impaired long-term potentiation and temporal summation of excitatory postsynaptic potentials in hippocampal slices (16). Loss of ASIC1a also eliminated hippocampal neuron currents evoked by pH 5 stimuli and markedly reduced currents generated by more extreme acidosis (6, 16, 17).

In previous studies, ASIC1a expression was abundant in the amygdala complex (6, 14, 25), which is required for fear conditioning and the expression of fear (4, 5, 26). Moreover, extracellular acidosis elicited a greater H^+-gated current density in amygdala neurons than in hippocampal neurons (6). ASIC1 null mice displayed deficits in cue and context fear conditioning, while baseline fear on the elevated plus maze remained intact. These studies suggested that ASIC1a and H^+-gated currents contribute to the neural mechanisms underlying fear conditioning. To test this hypothesis, we asked whether overexpressing ASIC1a would increase H^+-gated currents and enhance fear conditioning.

Materials and Methods

Transgenic (Tg) Mice. The plasmid vector was constructed by using a strategy described by Stec *et al.* (27). Briefly, the human *ASIC1a* cDNA, with the FLAG epitope sequence inserted immediately after the first ATG (16), was subcloned into pSTEC-2 (27). A PCR fragment containing the rat synapsin I promoter (a generous gift of Manfred Killiman, Ruhr-Universität, Bochum, Germany) was inserted upstream of the FLAG-*hASIC1a* sequence (28). A chimeric intron, composed of the 5′ splice site from the β-globin intron and the 3′ splice site from an *IgG* intron, was inserted between the *synapsin I* promoter and the first ATG of FLAG-*hASIC1a*, as described (27). An *Eco*RV restriction enzyme site was engineered by QuikChange mutagenesis (Stratagene) into the 5′ flanking sequence of the *synapsin I* promoter so that an *Eco*RV restriction enzyme digestion could be used to excise the entire transgene from the prokaryotic vector. It was then microinjected into one-cell fertilized mouse embryos obtained from superovulated C57BL/6J × SJL/J (B6SJL F2) mice by using standard procedures (29). Genotype of offspring was determined from tail DNA by using PCR and the following primers: 5′-TTC CCA TAC CGC GTG AAG ACC AC-3′ and 5′-GTC ATC GTC GTC CTT GTA GTC-3′. Tg lines were maintained by backcross breeding to C57BL/6.

The rate of transgene inheritance in lines 1 and 2 was 43% (n = 49) and 45% (n = 97), respectively, and Tg mice survived to adulthood. The overall size and appearance of Tg mice was normal, and there were no obvious signs of toxicity from overexpressing ASIC1a. All mice received standard mouse chow (LM-485, Teklad, Madison, WI) and water ad libitum. Care of the mice used in the experiments met the standard set forth by the National Institutes of Health in their guidelines for the care and use of experimental animals, and all procedures were approved by the University Animal Care and Use Committee at the University of Iowa.

Abbreviations: ASIC, acid-sensing ion channel; Tg, transgenic.

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Immunoblotting. Mouse brain protein lysate was prepared as described (6). Briefly, tissue was homogenized in lysis buffer, which contained PBS, aprotinin (40 μg/ml), leupeptin (40 μg/ml), pepstatin A (20 μg/ml), phenylmethanesulfonyl fluoride (40 μg/ml), and ethylenediamine tetraacetate (2 mM), by using a Dounce homogenizer (Wheaton Scientific). The homogenate was cleared of large particles with a 10-min centrifugation at 700 $\times$ *g*. Membrane proteins were precipitated at 170,000 $\times$ *g* for 30 min (Beckman TL-100, TLA-100 rotor). The pellet was resuspended in PBS with protease inhibitors. All steps in sample preparation were performed on ice or at 4°C. Protein concentration was determined by Lowry assay (30), and 50 μg was run on 8% acrylamide gel and western blotted. Signals were detected by enhanced chemiluminescence (Pierce). For deglycosylation, *N*-glycanase (Prozyme, San Leandro, CA) was used to deglycosylate 50 μg of mouse brain protein lysate. The sample was denatured at 100°C for 5 min and cooled to room temperature, and Nonidet P-40 was added to a final concentration of 0.75%. The reaction mixture was incubated overnight with 2 μl of *N*-glycanase (37°C) and western blotted. For immunoprecipitation, Triton X-100 (Pierce) was added to a final concentration of 1% to the cleared homogenate (described above). The sample was then further purified with a 1-min spin at 2,800 $\times$ *g*. The supernatant was incubated overnight at 4°C with 1 μl of anti-Flag antibody (Sigma) with shaking. It was then bound to 50 μl of protein A Sepharose for 30 min at 4°C. After three washes with cold PBS, the pellet was resuspended in sample buffer with 2% SDS, run on 8% acrylamide gels, and western blotted. The signal was detected by enhanced chemiluminescence (Pierce). The primary antibodies used in immunoblotting were anti-Flag (Sigma) at 1:2,000, anti-PSD-95 (Upstate, Charlottesville, VA) at 1:100,000, anti-actin (Santa Cruz Biotechnology) at 1:10,000, and anti-ASIC (MTY) antiserum at 1:15,000 (6). The MTY antibody is directed against an epitope common to the C-termini of both human and mouse ASIC1. Secondary antibodies were anti-rabbit IgG HRP (Amersham Pharmacia), anti-mouse IgG HRP (Amersham Pharmacia), and anti-goat IgG HRP (Jackson ImmunoResearch), all at 1:10,000.

Synaptosome Preparation. Sucrose gradients were prepared by layering 10 ml of 1.2, 1.0, and 0.85 M sucrose from bottom to top in a centrifuge tube (326823, Beckman) and allowing 2 h for settling. Mouse brain lysates (three per gradient) were prepared as described above. After the 170,000 $\times$ *g* centrifugation, the membrane pellet was resuspended in 2.5 ml of 0.32 M sucrose, in 1 mM $NaHCO_3$ with protease inhibitors. The resuspended membrane fraction was layered on the gradient, centrifuged at 82,000 $\times$ *g* for 120 min (Beckman L8–70M, SW28 rotor), and allowed to come to a stop without braking. The synaptosome-containing band (between 1.0 and 1.2 M sucrose) was collected (31), and the relative amount of ASIC1a protein in this fraction was compared to the amount in whole brain and total membrane fractions by Western blotting at 50 μg per lane.

Immunocytochemistry. Mouse hippocampal neurons were prepared as described (6) and plated on CC2 eight-well slides (Nalge Nunc). After 10–14 days, cultures were fixed in PBS with 4% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) and 0.25% Triton X-100 (Pierce) for 5 min. Slides were rinsed twice and blocked with 10% BSA in PBS for 30 min at room temperature. Affinity-purified MTY antibody (1:25) (6) and secondary antibody (Cy3-conjugated anti-rabbit IgG, 1:300, Jackson ImmunoResearch) were prepared in 3% BSA in PBS and applied for 2 h at room temperature and 1 h at 37°C, respectively. Slides were washed with PBS followed by distilled H_2O, mounted with VECTASHIELD (Vector Laboratories), and visualized by using a Bio-Rad MRC 1024 confocal microscope.

Whole-Cell Voltage-Clamp Experiments. Mouse hippocampal cultures were generated from postnatal day 1–2 pups as described (6). Whole-cell patch-clamp recordings were performed 7–14 days after seeding on neurons from at least two different litters as described (6, 17).

Immunohistochemistry. Fresh frozen coronal slices (7.5 μm) through the forebrain were prepared as described (6). Slices were postfixed in PBS with 4% formaldehyde/4% sucrose for 15 min, followed by 0.25% Triton X-100 in PBS for 5 min at room temperature. They were immunolabeled by using the TSA Fluorescence Systems (Perkin–Elmer) as described (6). Primary antibody was anti-Flag (Sigma) at 1:1,000, and secondary was anti-mouse IgG HRP (Amersham Pharmacia) at 1:200. Slices were mounted with VECTASHIELD and visualized with an Olympus (Melville, NY) BX-51 epifluorescence microscope equipped with Spot RT Slaer (Diagnostic Instruments, Sterling Heights, MI.)

Context Fear Conditioning. On day 1, naïve mice were placed in a conditioning chamber (MED Associates, St. Albans, VT) (6). After 3 min, animals received a foot-shock (1 sec, 0.5 mA) through an electric floor grid. Three foot-shocks were given, separated by 1-min intervals. Mice were then returned to their home cages. On day 2, mice were placed in the conditioning chamber for 15 min. Freezing, defined as the absence of movement, was scored from videotape during 1-min intervals by trained observers, blinded to genotype. To determine the sensory stimulus response threshold, naïve mice were placed in the test chamber, and sets of 10 foot-shocks were delivered starting at 0.01 mA. For each subsequent set, the foot-shock amplitude was increased by 0.005 mA. The number of responses per set of 10 shocks was defined as foot lifting or head twitching that coincided with shock delivery. To determine vocalization threshold, foot-shocks were delivered beginning at 0.08 mA. Subsequent foot-shocks were delivered with increasing current steps of 0.01 mA until vocalization occurred. For behavioral experiments the results did not differ between the two lines of Tg mice, therefore the data were combined. Mice used for these experiments were 15–20 weeks of age at the time of data collection. Non-Tg age and sex-matched littermates were used as controls.

Elevated Plus Maze. Naïve mice were placed in the center of the plus maze and allowed to roam freely for 5 min as described (6). Behavior was scored from videotapes by observers blinded to genotype.

Results

ASIC1a Was Overexpressed in Tg Mouse Brain. We generated Tg mice by using the pan-neuronal synapsin I promoter (28) cloned upstream of the human *ASIC1a* cDNA. We included a FLAG-epitope engineered into the 5′ coding sequence just after the first ATG. Two independent lines of Tg mice (Tg_1 and Tg_2) were generated. Allele-specific RT-PCR detected transgene expression in total brain RNA from both lines (Fig. 1*A*). Western blots of whole brain lysates with an antibody that detects mouse and human ASIC1 proteins revealed increased ASIC1 levels in both Tg lines (Fig. 1*B*). In Tg mice, the antibody detected the endogenous mASIC1a, plus a more slowly migrating species (Fig. 1 *B*, *C*, and *E*; m and h, respectively). Blotting with an antibody to the FLAG epitope identified the higher molecular mass species as the FLAG-tagged human ASIC1a protein (Fig. 1*C*).

We also tested the processing of hASIC1a and its interaction with endogenous mASIC1a. ASIC1 protein from both WT and Tg mice was sensitive to *N*-glycanase digestion (Fig. 1*D*), suggesting that the endogenous and transgenic proteins were similarly glycosylated and processed. To test whether transgenic and

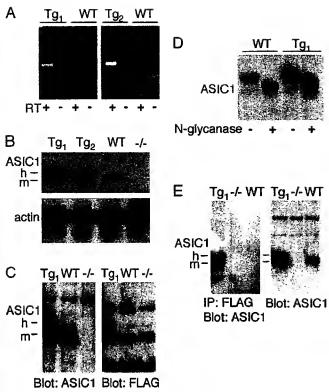

Fig. 1. FLAG-hASIC1 expression in Tg mouse brain. (*A*) RT-PCR analysis of transgene expression in total brain RNA from Tg_1, Tg_2, and WT mice. Experiments were performed with (+) or without (−) reverse transcriptase (RT). (*B*) Western blot analysis of ASIC1 expression in whole brain protein lysates from mice of indicated genotype. Blot was probed with antibody (MTY) directed against an epitope common to the C-termini of both mouse and human ASIC1. Actin immunoblotting was used to confirm equivalent protein loading. −/−, ASIC1 null animals. h and m, human and mouse ASIC1a, respectively. (*C*) Whole brain lysates were blotted with anti-ASIC1 antibody (MTY) or anti-FLAG antibody. Both blots were obtained from the same gel. Compared to Western blot in *B*, the electrophoresis time was extended for better resolution of the two ASIC1a species. (*D*) Whole brain lysates were treated with (+) or without (−) *N*-glycanase and blotted with MTY. (*E*) Whole brain lysates were immunoprecipitated with anti-FLAG antibody followed by immunoblotting with MTY (*Left*) or blotted with MTY without immunoprecipitation (*Right*).

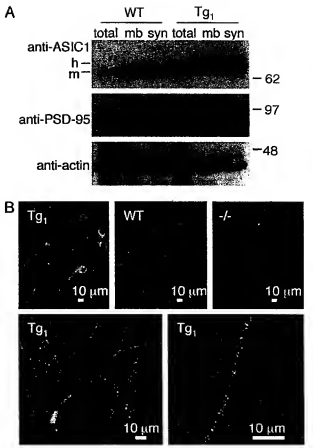

Fig. 2. Subcellular distribution of ASIC1 in WT and Tg mice. (*A*) Whole brain lysates (total), membranes (mb), and the synaptosome-containing brain fraction (syn) were western blotted with the indicated antibodies. (*B*) Cultured hippocampal neurons were prepared from mice with indicated genotypes and immunolabeled with affinity-purified anti-ASIC MTY antibody. (*Lower*) Immunolabeling of Tg_1 neurons at higher magnification. The bed of glial cells underlying the neurons showed no immunofluorescence.

endogenous ASIC1a formed multimers, we used the anti-FLAG antibody to immunoprecipitate FLAG-tagged hASIC1 from whole brain lysates and then blotted with the MTY antibody. The anti-FLAG antibody immunoprecipitated both transgenic and endogenous ASIC1a from Tg but not WT or ASIC1 −/− brain (Fig. 1*E*). These results indicate that transgenic hASIC1a formed a multimeric complex with mASIC1.

Transgenic ASIC1a Was Expressed in Neurons and Enriched in Synaptosomes. We used the Tg animals to test the hypothesis that ASIC1a protein is distributed in a pattern consistent with synaptic localization. Synaptosome-containing brain fractions revealed that ASIC1a protein coenriched with PSD-95 in both WT and Tg mice, although the abundance was greater in Tg brain (Fig. 2*A*). In hippocampal neurons from Tg mice, we found ASIC1a at the soma and distributed along dendrites in a punctate pattern (Fig. 2*B*). In contrast, we found little or no immunostaining of glial cells, which in these cultures form a dense bed on which the neurons settle. This result suggests that transgene expression was neuron-specific. There was little ASIC1a staining in WT neurons, consistent with our previous observation that endogenous ASIC1a has low levels of expression in the hippocampus (6). Together, the biochemical fractionation and immunolocalization data suggest that ASIC1a exists in the soma and neurites, where it may have a synaptic distribution.

ASIC1a Overexpression Augmented Central H^+-Evoked Currents. Previous studies proposed that ASIC1a was the subunit most important for determining the amplitude of H^+-gated current, whereas ASIC2a influenced kinetics, including the desensitization rate of the current (τ_D) (16–18). Supporting this hypothesis, we found that overexpressing ASIC1a doubled acid-evoked current density in Tg neurons (Fig. 3 *A* and *B*). This increase could result from more ASIC1a homomultimers or more heteromultimeric channels. τ_D might distinguish between these alternatives because currents from ASIC1a homomultimers desensitize more slowly than ASIC1a/2a heteromultimers (17, 32, 33). In ASIC1a transgenic neurons, we found a slowing of τ_D compared to WT neurons (Fig. 3 *A* and *C*). Although the proportion of homomultimeric and heteromultimeric channels remains uncertain, these data suggest that overexpressing ASIC1a increases current at least in part by producing more ASIC1a homomultimers. It is possible that the increase in current amplitude might be caused by a shift in the pH dose–response; however, this did not appear to be the case because the

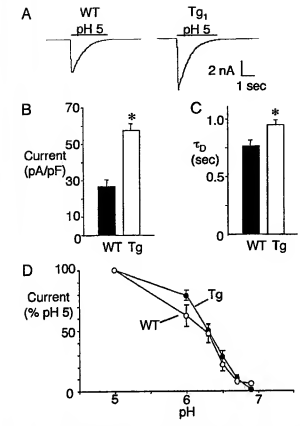

Fig. 3. Proton-gated currents in cultured hippocampal neurons from WT and Tg mice. (*A*) Representative traces from mice of indicated genotype. (*B*) Peak current density during pH 5 application ($n = 52$ for Tg; $n = 33$ for WT). *, $P \leq 0.001$ compared to WT by Student's t test. (*C*) Desensitization rate (τ_D) of currents evoked by pH 5 solution ($n = 41$ for Tg; $n = 22$ for WT). *, $P \leq 0.05$ compared to WT. (*D*) pH dose–response curve. Error bars represent SEM.

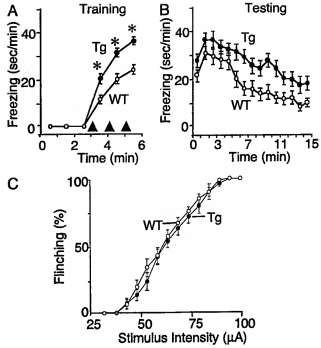

Fig. 5. Behavioral analysis of context fear conditioning. (*A*) Amount of time mice froze during 1-min intervals (sec/min) during training. The delivery of foot-shocks is indicated by arrowheads. Data from Tg1 ($n = 13$) and Tg2 ($n = 11$) mice were not statistically different ($P = 0.98$), and so were pooled. Data are mean ± SEM. *, $P < 0.0001$ compared to WT ($n = 25$ for WT; $n = 24$ for Tg). (*B*) Amount of time mice froze during 1-min intervals (sec/min) during testing. Tg mice spent more time freezing than WT animals ($P < 0.0001$ by linear mixed model analysis for repeated measures). (*C*) Percentage of foot-shocks that evoked a flinching response determined over a range of stimulus intensities. At each intensity, 10 foot-shocks were delivered ($n = 15$ for WT; $n = 17$ for Tg). $P = 0.49$ by logistic regression with the procedure GENMOD (SAS/STAT V.8.1, SAS Institute, Cary, NC).

pH-sensitivity of H^+-evoked currents was similar for WT and Tg neurons (Fig. 3*D*).

Transgenic ASIC1a Protein Was Abundant in the Amygdala and Hippocampus. To determine the distribution of hASIC1a expression, we immunolabeled coronal sections of forebrain with the anti-FLAG antibody. Although there were variations in the intensity, the staining pattern for the two Tg lines was similar, with the amygdala and hippocampus both showing abundant ASIC1a (Fig. 4). In the hippocampus, transgenic protein was distributed to the dendritic fields in both stratum radiatum and stratum oriens, consistent with the dendritic distribution observed in cultured neurons. In the amygdala, Tg1 brain showed preferential ASIC1a expression in the basolateral nucleus, and Tg2 expressed ASIC1a throughout the amygdala complex.

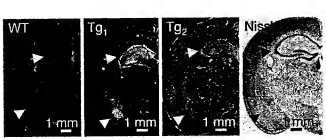

Fig. 4. Immunohistochemistry of coronal sections of forebrain from WT, Tg1, and Tg2 mice. Labeling with the anti-FLAG antibody is in white. The arrows point to hippocampal formation, and the arrowheads point to the amygdala complex. A Nissl-stained section from a WT mouse is included for orientation.

ASIC1a Overexpression Enhanced Context Fear Conditioning. We previously showed rich expression of mASIC1a in the amygdala and found that eliminating ASIC1 markedly reduced H^+-gated currents and impaired context and cued fear conditioning (6). Our present finding of abundant hASIC1a in the amygdala and increased acid-evoked currents in Tg animals raised the hypothesis that ASIC1a overexpression might have the opposite effect of ASIC1 disruption and enhance acquired fear. Under baseline conditions, neither WT nor Tg mice showed significant freezing behavior after the introduction into the test cage, suggesting that they were not afraid of the novel environment (Fig. 5*A*). After foot-shocks, both genotypes froze, but the duration of the behavior increased in Tg mice. This pattern persisted throughout the testing period (Fig. 5*B*). These results indicate that ASIC1a overexpression enhanced fear-related freezing behavior.

Because members of the ASIC family have been implicated in sensory function (10, 34–38), we asked whether the increased freezing in Tg mice might be due to increased ability to feel the shock. To test this, we did several studies. We counted the number of trials in which naïve mice flinched in response to foot-shock; the two genotypes showed similar flinching behavior at all stimulus intensities (Fig. 5*C*). We also assessed the vocalization threshold by gradually increasing the stimulus intensity until a vocalization was evoked; the vocalization threshold

was similar for WT (0.23 ± 0.01 mA; $n = 15$) and Tg (0.21 ± 0.01 mA; $n = 13$; mean ± SEM; $P = 0.8$) animals and was below the 0.5-mA stimulus used during conditioning. In addition, the average duration of hyperactivity after the 0.5-mA foot-shock delivered during fear conditioning did not differ in WT (1.7 ± 0.1 sec; $n = 25$) and Tg (1.8 ± 0.1 sec; $n = 25$; $P = 0.32$). Taken together, these data suggest that the increased freezing response in the Tg mice was likely not due to increased sensory function.

We also asked whether Tg mice have a difference in baseline anxiety that might explain the enhanced behavioral effect. To test this possibility, we used the elevated plus maze, which provides a measure of baseline fear, and scored several anxiety-related behaviors. The average time spent in the closed arms of the maze was similar for the two groups (WT, 172 ± 13 sec, $n = 9$; Tg, 180 ± 10 sec, $n = 8$; $P = 0.62$). The number of open arm entries was the same for both genotypes (WT, 14.4 ± 1.0; Tg, 13.6 ± 1.0; $P = 0.66$). And the amount of time spent inactive huddled in the closed arms was not significantly different (WT, 63.4 ± 11.3 sec; Tg, 64.3 ± 5.9 sec; $P = 0.95$). The normal activity of the Tg mice on the elevated plus maze suggests that ASIC1 overexpression did not affect baseline anxiety.

Discussion

The data described here, together with our previous studies of ASIC1 −/− mice indicate that ASIC1a plays an important role in fear-related freezing behavior. Endogenous mASIC1a was abundant in the amygdala (6, 14, 25), a key component of the circuitry for learned fear (4, 5, 26). It was also expressed in the cingulate cortex, nucleus accumbens, and other structures that may contribute to the emotional importance of external stimuli and/or the expression of fear (6, 39). Our studies of mouse behavior indicate that ASIC1a plays a role in the fear response; overexpressing ASIC1a enhanced fear conditioning, whereas eliminating ASIC1a reduced fear in this animal model of anxiety.

Our electrophysiological data support earlier suggestions that ASIC1a plays a key role in determining the amplitude of acid-evoked currents. Compared to WT, increasing ASIC1a expression doubled the current response to a pH 5 challenge, disrupting one of the two ASIC1 alleles cut the current in half (17), and eliminating ASIC1a abolished the response (6, 16, 17). However, central neurons also express ASIC2a and ASIC2b (11–13, 15, 17), which combine with ASIC1a to generate heteromultimeric channels (12, 17, 18, 32, 40). The pH-sensitivity, kinetics, and response to modulatory agents of heteromultimers differs from that of ASIC1a homomultimers (12, 17, 20, 23, 32, 40). At present, we do not know which properties of ASIC-mediated currents are most important in the fear response. However, the results presented here suggest that changing ASIC1a levels or activity could alter H^+-gated currents and consequently alter acquired fear. For example, transcriptional regulation might play an important role. In sensory neurons, inflammatory mediators such as serotonin, nerve growth factor (NGF), bradykinin, and interleukin 1 may increase ASIC expression and increase H^+-evoked currents (41). Interestingly, serotonin, NGF, and interleukin 1 have been implicated in fear conditioning in rodents (42–44) and may play an important role in anxiety in humans (45). ASIC1 activity might also be influenced by FMRFamide-like peptides (19, 21, 22, 46), which have been associated with fear-related avoidance learning in rodents (47). If polymorphisms or mutations in the *ASIC1* gene exist in humans, they might also affect H^+-gated currents and fear.

We cannot exclude the possibility that overexpressing ASIC1a could cause some toxicity, but many aspects of behavior were normal in the Tg mice. In fact, if overexpression were toxic to amygdala neurons, we would have expected to reduce freezing rather than enhance it (3, 4, 26). These data suggest that ASIC1a channels are predominantly closed until activated by ligand. However, it is possible that an increase in acid-activated currents could be toxic under pathological conditions such as ischemia and seizures, which are accompanied by extracellular acidosis (48–50). In addition, because the transgenic ASIC1a protein was widely expressed in the brain, it would not be surprising if behaviors other than the fear response were also affected.

We speculate that an increase in H^+-gated currents could represent a predisposition for the development of anxiety disorders such as posttraumatic stress disorder and panic disorder. For many years it has been known that inhaling CO_2 can trigger panic attacks in patients with panic disorder (51–53). In the brain, the reaction between CO_2 and water catalyzed by carbonic anhydrase rapidly generates H^+ and causes central acidosis. Perhaps acidosis triggers central H^+-gated currents and potentiates feelings of panic. The observation that neither ASIC1 disruption nor ASIC1a overexpression led to gross toxicity suggests that ASIC1a antagonists might represent a safe approach for reducing anxiety in the clinical setting.

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